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An Assay for X Inactivation Based on Differential Methylation at the Fragile X Locus, *FMR1*

Laura Carrel and Huntington F. Willard

Department of Genetics and Center for Human Genetics, Case Western Reserve University School of Medicine and University Hospitals of Cleveland, Cleveland, Ohio

We describe an assay analyzing methylation at the fragile X mental retardation gene, *FMR1*, to examine patterns of random or non-random X chromosome inactivation. Digestion of genomic DNA with the methylation-sensitive enzyme HpaII cleaves two restriction sites near the CGG repeat of the *FMR1* gene if they are unmethylated on the active X chromosome, but fails to digest these sites on the inactive chromosome. Subsequent PCR using primers that flank the sites and the variable CGG repeat within the *FMR1* gene amplifies alleles only on undigested, methylated inactive X chromosomes. Amplification of the hypervariable CGG repeat distinguishes alleles in heterozygous samples, while the relative ratio of alleles within a *Hpa*II-digested sample reflects the randomness or non-randomness of inactivation. To demonstrate that methylation of the HpaII sites within the amplified *FMR1* fragment correlates strictly with the activity state of the X chromosome, we have tested the validity of this assay by comparing DNA from normal males and females, as well as DNA from mouse/human somatic cell hybrids carrying either active or inactive human X chromosomes. The data demonstrate that this assay provides a reliable means of assessing the inactivation status of X chromosomes in individuals with X-linked disorders or X chromosome abnormalities.

KEY WORDS: X inactivation, DNA methylation, FMR1

INTRODUCTION

Early during female development, one X chromosome in each cell is randomly inactivated as a means of dosage compensation [reviewed in Brown and Willard, 1993]. This random choice generally results in approximately equal numbers of cells that have inactivated either a paternal or maternal X chromosome, and this pattern is maintained through successive cell divisions. Assays that determine the degree of randomness and document and/or quantitate deviations from randomness are important for the assessment of skewed inactivation patterns in females expressing X-linked diseases or for determining carrier status in several X-linked disorders where preferential inactivation reflects cell selection to prevent expression of a mutant allele [reviewed in Willard, 1995]. Additionally, clonality of tumors can be evaluated in females [Vogelstein et al., 1987; Lee et al., 1994] since clonal tumors necessarily reflect the X inactivation status (and concomitant DNA methylation pattern) of the single cell from which they are derived. Numerous X inactivation assays have been described, some using conventional polymorphisms [Vogelstein et al., 1987; Maestrini et al., 1992] and others more informative VNTR [Boyd and Fraser, 1990] or microsatellite [Allen et al., 1992; Hendriks et al., 1992] markers. However, because these tests require allelic differences to distinguish the two X chromosomes, new assays at highly polymorphic loci are desirable. This report describes an X inactivation assay at the fragile X mental retardation locus, *FMR1*, in Xq27.3.

The *FMR1* gene is subject to X chromosome inactivation [Kirchgessner et al., 1995] and methylation of *FMR1* at the 5′ end and the CGG repeat within the gene has been well studied [Hansen et al., 1992; Hornstra et al., 1993; Luom et al., 1993], demonstrating that CpG sites on the inactive X chromosome are generally methylated, whereas they are unmethylated on active X chromosomes (in males or females). This differential methylation forms the basis for the X inactivation assay, in combination with the polymorphic CGG repeat. In this report, we describe an allele-specific methylation assay and document its ability to discriminate between active and inactive X chromosomes by using a series of mouse/human somatic cell hybrids that contain human X chromosomes of either type.

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Address reprint requests to H.F. Willard, Department of Genetics, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106.

MATERIALS AND METHODS

Cell Lines and DNA Preparation

Lymphoblast cell lines are from NIGMS Human Genetic Mutant Cell Repository, with the exception of AG [Pettigrew et al., 1991] and VTL 754 [Siu et al., 1990], which were provided by Dr. D. Ledbetter and Dr. R. Nussbaum, respectively (NCHGR, NIH, Bethesda, MD). All mouse/human somatic cell hybrids have been previously described [Willard et al., 1993]. Genomic DNA from established cell lines was prepared by phenol extraction or by a salting out procedure [Miller al., 1988]. DNA from peripheral blood lymphocytes was isolated with a DNA extraction kit from Oncor, Inc.

FMR1 Methylation Assay

Primers c (5′ GCTCAGCTCCGTTTCGGTTTCACTT CCGGT 3′) and f (5′ AGCCCCGCACTTCCACCACCA GCTCCTCCA 3′) were previously described [Fu et al., 1991]. PCR reactions were performed using 100 ng of genomic DNA either uncut or predigested to completion with HpaII. Digested samples were heat treated to inactivate the restriction enzyme. PCR conditions are modified from [Fu et al., 1991]. FMR-c was 5′ end-labeled with ^{32}P dATP [Sambrook et al., 1989], column purified (Nuctrap, Stratagene) to remove unincorporated nucleotides, and 2×10^5 cpm, corresponding to about 1–2 pmol, was added to each reaction with 8 pmol of unlabeled FMR-f. Reactions were performed in 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 200 μM dATP, 200 μM dTTP, 200 μM dCTP, 50 μM dGTP, 150 μM 7-deaza-dGTP, 10% DMSO, and 1 U Ampli-Taq polymerase (Perkin-Elmer). After an initial denaturation at 95°C for 4 min, samples were amplified for 30 cycles of 1.5 min at 95°C, 1 min at 65°C, and 2 min at 72°C, in an Ericomp water-cooled thermocycler. Aliquots of each PCR reaction were mixed 2:1 with formamide sequencing dye, denatured, and analyzed on 6% denaturing polyacrylamide gels. Markers (1 kb ladder, Gibco-BRL) were also end-labeled to run concurrently as a size standard. Dried gels were exposed with intensifying screens generally for 1 to 2 days. The relative intensity of bands was evaluated by phosphorimaging analysis or, in preliminary experiments, by visual inspection using dilution standards.

RESULTS AND DISCUSSION

The assay described here applies principles established previously for methylation assays [e.g., Vogelstein et al., 1987; Lee et al., 1994; Allen et al., 1992] and uses two HpaII sites that flank the highly variable CGG repeat at the 5′ end of the *FMR1* gene [Fu et al., 1991] (Fig. 1). These sites have methylation patterns that correlate with the activity of the chromosome, such that at least one site is always unmethylated on the active X chromosome, but in at least the majority of cells, both sites are methylated on the inactive X chromosome [Hornstra et al., 1993; Luom et al., 1993]. Digestion of genomic DNA with the methylation-sensitive enzyme HpaII cuts these sites on the unmethylated active X. Subsequent PCR, using primers that encompass the restriction sites as well as the polymorphic CGG repeat, specifically amplifies alleles that are methylated and therefore undigested by the HpaII treatment.

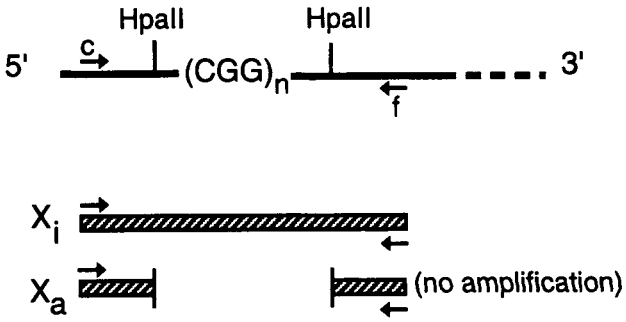

Fig. 1. Methylation assay of X inactivation based on PCR analysis of 5′ untranslated portion of the *FMR1* gene. HpaII is unable to cut the methylated sites on the inactive X chromosome (X_i), allowing subsequent amplification. However, digestion at either or both sites on the active X chromosome (X_a) prevents PCR.

In female samples, the two X chromosomes can be differentiated by fragment size variations due to polymorphism in the number of CGG repeats [Fu et al., 1991]. DNA samples from males with only an active X chromosome do not amplify if the genomic DNA is predigested with HpaII (Fig. 2). However, females with both an active and inactive X show a product, presumably from the methylated, inactive X chromosome (Fig. 2). In female samples, the relative representation of each allele in the HpaII-digested samples (as compared to the amplified undigested samples, which controls for potential amplification variability between alleles due to size) reflects the randomness or non-randomness of X inactivation in that sample.

For example, for two of the females shown in Figure 2, the *FMR1* alleles in the samples pre-digested with HpaII are of differing intensity, indicating skewed inactivation. Image analysis of these samples (3 and 4) revealed ratios of ~25:75 and ~40:60, indicating moderate non-random inactivation in each case. These skewed patterns have been confirmed by other methylation assays at the *MAOA* and *AR* loci [Allen et al., 1992; Hendriks et al., 1992], as well as by a direct assay of X chromosome expression based on a transcribed polymorphism at the *XIST* locus [Rupert et al., 1995]. The third female sample shown in Figure 2 (sample 5) is from a female with a balanced X;19 translocation who shows complete non-random X inactivation as expected, since the normal X in this cell line is always late-replicating [Mohandas et al., 1980] and, since in most balanced X;autosome translocations, the normal X is selectively inactivated to maintain proper dosage [Therman and Patau, 1974; Willard, 1995].

The quantitative aspects of this assay can clearly be improved, by use of fluorescent primers and automated gel scanning and/or by incorporation of a second, control locus into each PCR reaction. Nonetheless, the current assay, quantitated either visually or by image analysis, provides clear discrimination of random vs. skewed X inactivation patterns. While improved quantitation may be desirable in some instances, there is no indication at present that there is any clinical signifi-

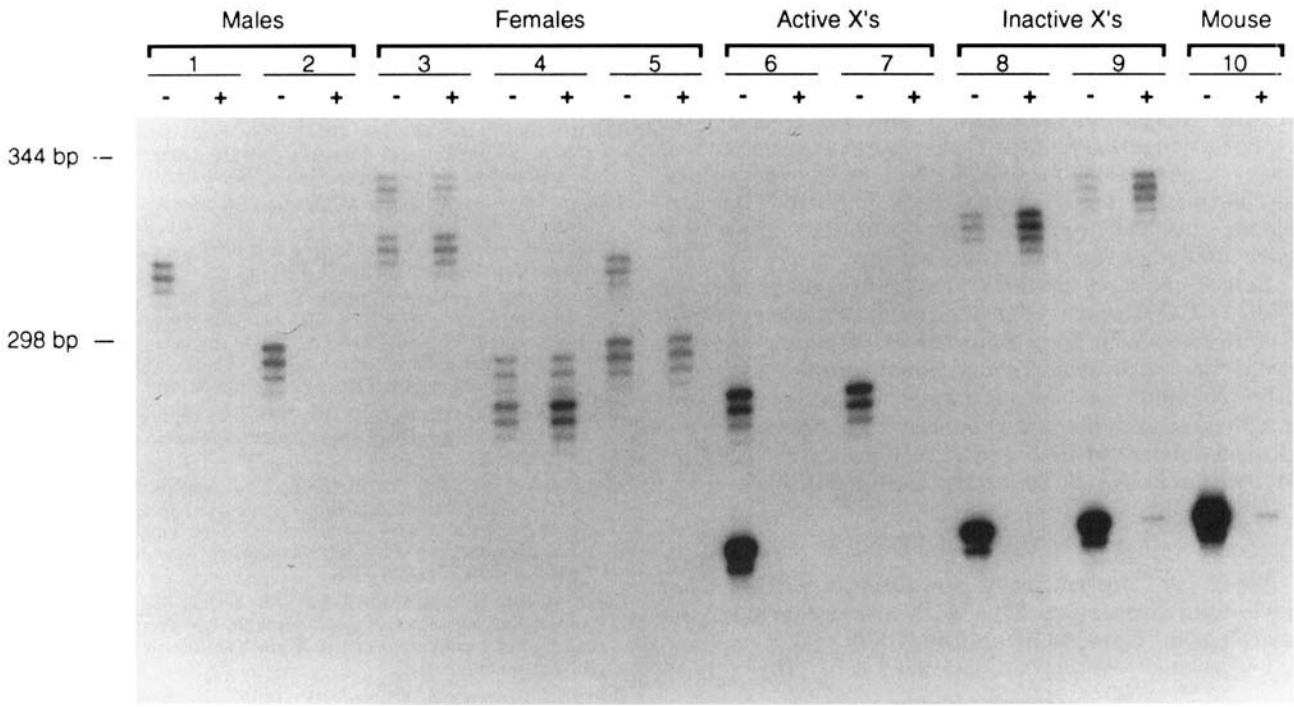

Fig. 2. Representative results of *FMR1* methylation assay of X inactivation. For each sample, a + or − indicates whether the genomic DNA has been digested with HpaII before amplification. Samples 1–4, normal male or female DNA isolated from peripheral blood lymphocytes; sample 5, fibroblast cell line GM0089 from a 46,X,t(X;19) female; samples 6 and 7, somatic cell hybrids containing an active human X chromosome; samples 8 and 9, somatic cell hybrids containing an inactive human X chromosome; sample 10, mouse A9. The most common allele size of 29 CGG repeats corresponds to a 308 bp PCR product. In the somatic cell hybrids, the intense bottom band is the mouse *Fmr1* allele, which also has unmethylated HpaII sites. Absence of this band in the hybrid AHA-11aB1 (sample 7) indicates that this hybrid has deleted or mutated a portion of the mouse *Fmr1* gene.

cance to relatively minor differences in X inactivation patterns (e.g., 40:60 vs. 30:70). Thus, classification of samples into "random," "moderately skewed," and "extremely skewed" patterns may be sufficient for most purposes.

The validity of this methylation assay was confirmed by examining DNA from normal males and females, three somatic cell hybrids carrying active human X chromosomes and four with inactive X's [Willard et al., 1993], and cell lines that were known [on the basis of published late-replication data; Mohandas et al., 1980; Siu et al., 1990] to be characterized by non-random X inactivation (Table I). In all cases, the data obtained were consistent with the expected or known patterns of

TABLE I. Summary of X Inactivation Assay Results*

Samples	Karyotype	DNA source	Amplification after *Hpa*II digestion	X inactivation pattern as determined by *FMR1* assay
Normal males (3)	46,XY	Human lymphocyte	−	Active X
AG	45,X/46,X,idic(X) (pter→q13:: q13→pter)	Human lymphoblast	−	Normal X is active in 100% of cells idic(X) does not contain copy of *FMR1* gene
Normal females (2)	46,XX	Human lymphocyte	+	Random X inactivation
Normal female	46,XX	Human lymphocyte	+	Skewed X inactivation (~75:25)
GM2859A	46,X,t(X;11)	Human fibroblast	+	>95% non-random inactivation
GM0089	46,X,t(X;19)	Human fibroblast	+	100% non-random inactivation
VTL754	46,X,t(X;13)	Human lymphoblast	+	100% non-random inactivation
AHA11aB1	Active X	Somatic Cell Hybrid	−	Active X
A23-1aC15	Active X	Somatic Cell Hybrid	−	Active X
t60-12	Active X	Somatic Cell Hybrid	−	Active X
Lt23-1E2Buv 5C126-7A2	Inactive X	Somatic Cell Hybrid	+	Inactive X
t11-4Aaz5	Inactive X	Somatic Cell Hybrid	+	Inactive X
t86-B1maz1b-3a	Inactive X	Somatic Cell Hybrid	+	Inactive X
t75-2maz34-4a	Inactive X	Somatic Cell Hybrid	+	Inactive X

*Established cell lines were from the following sources: AG [Pettigrew et al., 1991]; GM 2859A and GM0089 (NJGMS), VTL 754 [Siu et al., 1990]; somatic cell hybrids containing active or inactive human X chromosomes [Willard et al., 1993].

X inactivation and/or methylation (Fig. 2, Table I). In particular, no amplification was detected after HpaII digestion of alleles on the active X in hybrids (samples 6 and 7), whereas amplification of inactive X alleles was readily detectable (samples 8 and 9). Overall, the methylation pattern of the HpaII sites examined in this assay appears to be maintained in a number of tissues, as peripheral blood lymphocytes, cultured lymphoblasts, and cultured fibroblasts were examined and gave similar or expected assay results.

Since ~65% of females are heterozygous for the *FMR1* CGG repeat [Fu et al., 1991], this assay should be informative for X chromosome inactivation in a high proportion of cases and complements similar assays at other X-linked loci. In our hands, the assay is reproducible and reliable and is currently being used in our clinical laboratories to evaluate females referred for analysis of known or suspected X-linked disorders.

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